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Amphiphilic degradable polymers for immobilization and sustained delivery of sphingosine 1-phosphate

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ABSTRACT

Controlled delivery of the angiogenic factor sphingosine 1-phosphate (S1P) represents a promising strategy for promoting vascularization during tissue repair and regeneration. In this study, we developed an amphiphilic biodegradable polymer platform for the stable encapsulation and sustained release of S1P. Mimicking the interaction between amphiphilic S1P and its binding proteins, a series of polymers with hydrophilic poly(ethylene glycol) core and lipophilic flanking segments of polylactide and/or poly(alkylated lactide) with different alkyl chain lengths were synthesized. These polymers were electrospun into fibrous meshes, and loaded with S1P in generally high loading efficiencies (>90%). Sustained S1P release from these scaffolds could be tuned by adjusting the alkyl chain length, blockiness and lipophilic block length, achieving 35–55% and 45–80% accumulative releases in the first 8 h and by 7 days, respectively. Furthermore, using endothelial cell tube formation assay and chicken chorioallantoic membrane assay, we showed that the different S1P loading doses and release kinetics translated into distinct pro-angiogenic outcomes. These results suggest that these amphiphilic polymers are effective delivery vehicles for S1P and may be explored as tissue engineering scaffolds where the delivery of lipophilic or amphiphilic bioactive factors is desired.

1. Introduction

Angiogenesis is essential for tissue development, function, maintenance, repair and regeneration [1–5], and impaired angiogenesis due to either injuries or diseases can severely impair these processes. For instance, disruption of vascular network as a result of orthopedic trauma compromises the ability to vascularize bone grafts, resulting in high clinical failure rates of bone graft-mediated repair of traumatic bone defects [6]. In pathological conditions such as diabetes, the microangiopathic complication/tissue ischemia also retards bone injury repair and graft healing [7,8] as it disrupts the tightly coupled osteogenesis and angiogenesis processes [9]. Therefore, therapeutic strategies for promoting angiogenesis, particularly the formation of functional and stable vascular network, have long been sought after in scaffold-assisted tissue repair and regeneration.

Angiogenesis is a dynamic cascade of cellular and molecular events [10,11] involving the early stage of lumen formation (e.g. increased blood vessel permeability, basement membrane degradation, endothelial cell (EC) migration, proliferation and further assembly into tubular structure) and the later stage of nascent EC tube stabilization and maturation (e.g. mural cell recruitment and new basement membrane deposition). The entire angiogenesis process is tightly regulated by a dynamic balance of pro-angiogenic factors and vessel-stabilizing factors [12]. Current strategies for recapitulating this process in situ [5,13,14] involve the delivery of angiogenic stimuli, of which angiogenic growth factors, such as vascular endothelial growth factor (VEGF), are the most intensively studied [15–17]. VEGF is a potent angiogenesis initiator that is also known to disrupt pericyte coverage and inhibit subsequent vessel stabilization [18], thus the delivery of exogenous VEGF alone often results in sub-optimal neovascularization characterized with immature "leaky" vessels. Therefore, the delivery of alternative/complementary signaling molecules promoting the formation of a more extensive, stable and functional vascular network is highly desired. The phospholipid sphingosine 1-phosphate (S1P) has emerged as a highly promising candidate because of its dual role as an angiogenic stimulant and a blood vessel stabilizer.

During the early stages of angiogenesis, S1P acts as a potent EC chemoattractant [19,20], promoting EC proliferation, migration [21] and further assembly into tubes [22], while S1P receptor 1

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(S1P1) negatively regulates vessel sprouting to prevent excessive sprouting [23,24]. In the later stages of angiogenesis, S1P regulates vasculature remodeling and maturation by recruiting vascular smooth muscle cells and pericytes [25–28]. The local delivery of S1P or the S1P analogue FTY 720 has been shown to enhance wound healing in diabetic rats [29], stimulate blood flow in ischemic limbs [30], and promote calvarial bone formation [31–33] and allograft incorporation [34,35]. These studies support the potential benefit of the delivery of S1P in improving the functional outcome of tissue repair.

A significant challenge for translating the S1P-based pro-angiogenic strategy to successful tissue repair is, however, the lack of a tunable sustained release system enabling the optimization of its release kinetics for maximal stimulation of vessel formation and maturation. Scaffolds being explored for S1P encapsulation include hydrophobic poly(lactic-co-glycolic acid) thin films [31,32]/microspheres [30] and hydrophilic polyethylene glycol (PEG)-based hydrogels cross-linked by albumin [36]. While some of these scaffolds exhibited acceptable S1P loading efficiency, their intrinsic structures have limited direct interactions with the amphiphilic S1P, hence the tunability of the S1P release kinetics. Recently a cellulose hollow fiber-based system enabling timed delivery of S1P following earlier release of VEGF was shown to result in greater recruitment of ECs and a higher maturation index of formed vessels in a Matrigel plug model [37]. However, this delivery system required external manual regulation, which complicates its implementation for in vivo tissue regeneration. Overall, synthetic scaffolds demonstrating significantly improved S1P loading efficiency and more tunable S1P release kinetics are still lacking.

Structurally, S1P is an amphiphilic lysophospholipid composed of a zwitterionic headgroup and a hydrophobic 18-carbon (C18) aliphatic tail. In circulating blood, S1P is released from platelets [38] in micromolar concentrations, and most of the released S1P is stored by binding with albumin [20,39] and lipoproteins such as high-density lipoprotein (HDL) [40,41]. Recent structural studies revealed that the interaction of S1P with HDL is mediated by HDL-associated apolipoprotein M (apoM). Specifically, apoM was shown to have an amphiphilic binding pocket with a polar entrance to grab the hydrophilic S1P headgroup and an inner lipophilic pocket to accommodate the C18 aliphatic tail [42,43]. This amphiphilic interaction pattern is also observed with the bindings of S1P antagonist with S1P1 receptor [44] and S1P with S1P antibody [45]. Mimicking this interaction, we hypothesize here that an amphiphilic polymer scaffold incorporating both hydrophobic and hydrophilic segments could effectively bind S1P, translating into improved S1P loading efficiency. Furthermore, the release kinetics of the encapsulated S1P could possibly be tuned by adjusting the lipophilicity of the polymer. In this study, we test these hypotheses with a Poly(lactic acid) (PLA)–PEG–PLA (PELA)-based amphiphilic block copolymer platform [46] by incorporating alkylated lactides. By varying the alkyl side chain lengths, blockiness and block lengths, we examine their impact on the encapsulation, release and angiogenic outcome of S1P delivery using a combination of EC tube formation assay and ex vivo chicken chorioallantoic membrane (CAM) assay.

2. Materials and methods

2.1. Materials and general instrumentation

S1P was purchased from Cayman Chemical (Ann Arbor, MI). Growth-factor-reduced Matrigel was obtained from BD Biosciences (Bedford, MA). Fertile chicken eggs were supplied by Charles River Labs (Wilmington, MA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), and were used as received unless otherwise stated. 2-Hydroxyhexadecanoic acid was synthesized from 2-bromohexadecanoic acid as per the published protocols [47].

NMR spectra were recorded on a Varian INOVA-400 spectrometer. Molecular weights and polydispersity of polymers were determined by gel permeation chromatography (GPC) on a Varian Prostar HPLC system equipped with two 5 mm PLGel MiniMIX-D columns and a PL-ELS2100 evaporative light scattering detector. Calibrations were performed with polystyrene standards (polymer laboratories). Tetrahydrofuran was used as the eluent at a flow rate of 0.3 ml min^{-1}.

2.2. Design rationale of the amphiphilic polymers and alkylated lactide monomers

The PELA-based amphiphilic copolymer-based platform was designed to enable interactions between the polar S1P headgroup and the hydrophilic PEG segment, as well as between the lipophilic S1P tail and the hydrophobic PLA blocks. By inserting alkylated polylactides into PELA either in discrete blocks between the PEG core and the PLA ends or randomly with the PLA blocks, we hoped to further enhance S1P binding via hydrophobic interactions between the aliphatic side chains and the S1P lipid tail (Fig. 1A). It is worth noting that complete elimination of PLA from the amphiphilic copolymers (i.e. substituting two PLA blocks in PELA with alkylated polylactides) tended to result in liquids with lower molecular weight (Table S1) that are unsuitable for electrospinning fabrication of bulk scaffolds. Three distinct design elements were altered to allow the scaffolds to interact with S1P with varied affinities: the alkyl side chain lengths (C6 vs. C14), distribution (random copolymers with alkyl side chains spreading out vs. block copolymers with the alkyl side chains more densely clustered) and presentation density (low, medium and high alkylated repeating units relative to the PEG core).

The design of 3-methyl-6-alkyl-1,4-dioxane-2,5-diones as alkylated lactide monomers was motivated by their biocompatible degradation products, α-hydroxyl fatty acids, which are present in plants and mammals [48,49]. The choice of mono- instead of bialkylated lactides was due to the concern that the excessive steric hindrance of the latter may compromise the ring-opening polymerization efficiency.

2.3. Monomer syntheses

2.3.1. 3-Methyl-6-hexyl-1,4-dioxane-2,5-dione (C_6LA)

The monomer synthesis was carried out using a protocol modified from the literature [47,50,51]. To an ice-bath-chilled acetone solution (150 ml) of 2-hydroxyoctanoic acid (5.0 g, 31.21 mmol) and Et_3N (8.71 ml, 62.42 mmol) was slowly added 2-bromopropionyl bromide (3.43 ml, 32.77 mmol). The white suspension was then stirred at room temperature for 0.5 h before being filtered. The white residue obtained was washed twice more with acetone to give a combined light yellow filtrate of a total volume of 300 ml, to which was added Et_3N (8.71 ml, 62.42 mmol). The mixture was stirred at 65 °C for 2 h before it was cooled to room temperature and concentrated to 50 ml under reduced pressure. The concentrate was filtered, further concentrated and diluted with a mixture of *n*-hexane and EtOAc (*n*-hexane/EtOAc = 3/1, 150 ml), then passed through a short silica gel column to give the crude product, which was recrystallized twice with *n*-hexane to yield a white solid racemic monomer (1.85 g, 27.7% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.02 (m, 1H), 4.90 (m, 1H), 2.00 (m, 2H), 1.67 (m, 3H), 1.61 (m, 2H), 1.52 (m, 6H), 0.88 (m, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.77, 167.15, 77.11, 76.03, 72.73, 72.48, 32.15, 31.68, 31.62, 30.23, 28.97, 28.74, 24.84, 24.52, 22.71, 22.68, 17.77, 16.07, 14.24, 14.21 ppm.